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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68, C07H 21/04		A1	(11) International Publication Number: WO 96/41012 (43) International Publication Date: 19 December 1996 (19.12.96)
(21) International Application Number: PCT/US96/09637 (22) International Filing Date: 6 June 1996 (06.06.96)		(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(30) Priority Data: 08/474,450 7 June 1995 (07.06.95) US		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(60) Parent Application or Grant (63) Related by Continuation US 08/474,450 (CIP) Filed on 7 June 1995 (07.06.95)			
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(54) Title: UNIVERSAL PRIMER SEQUENCE FOR MULTIPLEX DNA AMPLIFICATION

(57) Abstract

The present invention provides primers that allow simultaneous amplification of multiple DNA target sequences present in a DNA sample. Further provided are methods for detecting multiple defined target DNA sequences in a DNA sample. Methods for high-throughput genetic screening are also provided. In yet another aspect, the present invention provides single-stranded oligonucleotide DNA primers for amplification of a target DNA sequence in a multiplex polymerase chain reaction.

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UNIVERSAL PRIMER SEQUENCE FOR MULTIPLEX DNA AMPLIFICATION

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Field of the Invention

This invention pertains to universal primers having use in amplification of DNA sequences by methods such as polymerase chain reaction (PCR), specifically to primers that allow the simultaneous amplification of a multiplicity of DNA sequences.

Background of the Invention

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Polymerase chain reaction (PCR) is a method whereby virtually any DNA sequence can be selectively amplified. The method involves using paired sets of oligonucleotides of predetermined sequence that hybridize to opposite strands of DNA and define the limits of the sequence to be amplified. The oligonucleotides prime multiple sequential rounds of DNA synthesis catalyzed by a thermostable DNA polymerase. Each round of synthesis is typically separated by a melting and re-annealing step, allowing a given DNA sequence to be amplified several hundred-fold in less than an hour (Saiki et al., *Science* 239:487, 1988).

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With the rapid advances in mammalian molecular genetics, an ever increasing number of disease genes have been identified. Accordingly, PCR has gained widespread use for the diagnosis of inherited disorders and the susceptibility to disease. Typically, the region of interest is amplified from either genomic DNA or from a source of specific cDNA encoding the cognate gene product. Mutations or polymorphisms are then identified by subjecting the amplified DNA to analytical techniques such

as DNA sequencing, hybridization with allele-specific oligonucleotides (ASOs), oligonucleotide ligation, restriction endonuclease cleavage or single-strand conformational polymorphism (SSCP) analysis.

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For the analysis of small genes and transcripts, or genes where the mutant allele or polymorphism is well characterized, amplification of a single defined region of DNA is sometimes sufficient. When analyzing large genes and transcripts or undefined genes, however, multiple individual PCR reactions are often required to identify critical base changes or deletions. Thus, to streamline the analysis of large complex genes, multiplex PCR (i.e., the simultaneous amplification of different target DNA sequences in a single PCR reaction) has been utilized.

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The results obtained with multiplex PCR are, however, frequently complicated by artifacts of the amplification procedure. These include "false-negative" results due to reaction failure and "false-positive" results such as the amplification of spurious products, which may be caused by annealing of the primers to sequences which are related to, but distinct from, the true recognition sequences.

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For use in multiplex PCR, a primer should be designed so that its predicted hybridization kinetics are similar to those of the other primers used in the same multiplex reaction. While the annealing temperatures and primer concentrations may be calculated to some degree, conditions generally have to be empirically determined for each multiplex reaction. Since the possibility of non-specific priming increases with each additional primer pair, conditions must be modified as necessary as individual primer sets are added. Moreover, artifacts that result from competition for resources (e.g., depletion of primers) are augmented in multiplex PCR, since differences

in the yields of unequally amplified fragments are enhanced with each cycle.

• 5 Weighardt et al. (PCR Meth. App. 3:77, 1993)
• describe the use of 5'-tailed oligonucleotides for PCR.
• However, a key feature of this amplification method
• involves separate annealing and primer extension reactions
• for each primer, which is not practical in a multiplex
• context. Therefore, complete optimization of the reaction
• 10 conditions for multiplex PCR can become labor intensive and
• time consuming. Since different multiplex PCRs may each
• have unique reaction conditions, development of new
• diagnostic tests can become very costly.

15 Thus, there is a need in the art for primers that allow multiplex PCR reactions to be designed and carried out without elaborate optimization steps, irrespective of the potentially divergent properties of the different primers used. Furthermore, there is a need in the art for primers that allow multiplex PCR reactions that, under the same reaction conditions, simultaneously produce equivalent amounts of each of many amplification products.

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Summary of the Invention

Multiple cycles of melting, reannealing, and DNA synthesis (i.e., a PCR reaction) are thereafter performed with the above mentioned DNA sample and invention oligonucleotide primers. Amplified target sequences may then be detected by any method, including, for example, hybridization with allele-specific oligonucleotides, restriction endonuclease cleavage, or single-strand conformational polymorphism (SSCP) analysis.

10 The invention also encompasses a method for detecting multiple defined target DNA sequences in a DNA sample. This method is carried out by performing the same procedure set forth above, in which the 3' sequence of one primer of each pair comprises a target DNA sequence itself or its complement. The method includes a further step of 15 detecting the amplification products, preferably by gel electrophoresis. In this embodiment, the presence or absence of an amplification product is diagnostic of the presence or absence of the target sequence in the original 20 DNA sample.

25 In another aspect, the invention encompasses methods for high-throughput genetic screening. The method, which allows the rapid and simultaneous detection of multiple defined target DNA sequences in DNA samples obtained from a multiplicity of individuals, is carried out by simultaneously amplifying many different target 30 sequences from a large number of patient DNA samples, using oligonucleotide primer pairs as above.

35 In yet another aspect, the present invention provides single-stranded oligonucleotide DNA primers for amplification of a target DNA sequence in a multiplex polymerase chain reaction. The primers have the structure 5'-XY-3', wherein X comprises an invention primer sequence, and Y comprises a sequence contained within or flanking a target sequence or its complement. Typically, Y comprises

a sequence from 17 to 25 bases in length, and the melting temperature of hybrids between the primers and their complements is at least 72°C.

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The methods and compositions of the present invention can be applied to the diagnosis of genetic and infectious diseases, gender determination, genetic linkage analysis, and forensic studies.

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Brief Description of the Drawings

Figure 1 is a table listing amplicon-specific oligonucleotide primer sequences.

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Figure 2 shows agarose gel analysis of primer concentration and annealing temperature for amplification of CFTR exon 21.

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Figure 3 shows agarose gel analysis of CFTR 15-plex amplicon organization.

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Figure 4 shows gel comparison of chimeric primers with sequence-specific primers for CFTR 15-plex PCR amplification.

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Figure 5 shows the results from multiple gene loci amplifications performed in a single PCR thermal cycler under identical reaction conditions and cycling parameters.

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Figure 6 shows the results from multiple gene loci amplifications performed in a single PCR thermal cycler under identical reaction conditions and cycling parameters.

Detailed Description of the Invention

5 Invention methods described herein provide an amplification system for multiplex PCR which is based on the use of chimeric primers tagged on the 5' end with an unrelated 20 nucleotide sequence (UPS). The examples provided below demonstrate that multiple genomic sequences can be co-amplified under identical reaction conditions and cycling parameters with very little optimization of PCR 10 conditions. Using invention compositions and methods, highly specific and efficient amplification of target sequences can be easily and reproducibly achieved by simple adjustment of the individual primer concentrations, with no additional modification of either the reaction components 15 or annealing temperatures.

20 Invention chimeric primers produced clean visibly detectable PCR profiles over an 8-fold range of template DNA concentrations (see examples). Moreover, the high level of consistency, with respect to the relative band 25 intensities, increases the informativeness and simplifies the interpretation of the results.

30 The enhanced specificity and efficiency which is conferred by using invention chimeric primers is due to a normalization of the hybridization kinetics. During the early rounds of the PCR, molecules are synthesized which contain the tagged primers at their 5' ends. Therefore, in all subsequent rounds of amplification, the amplicons 35 synthesized will all have identical 20 bp priming sequences with the predicted hybridization kinetics of the universal primer sequence (UPS) tag. Further, by virtue of the extremely GC-rich region at the 5' end of invention UPS sequences, under PCR conditions optimal for the UPS tag, nucleation is progressing in a primer oriented 5' to 3' direction. In addition, each chimeric primer pair has a 3' sequence-specific region with an internal stability profile

(ΔG) for primer duplexing lower than the UPS tag. Therefore, chimeric primers, which have an overall T_m significantly greater than 72° C serve as highly efficient yet stringent recognition sequences for the subsequent rounds of the PCR.

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Through normalizing hybridization kinetics, invention compositions and methods have largely eliminated the need to evaluate diverse reaction conditions and cycling parameters. Therefore, the use of invention UPS-tagged primers provides a less costly method in terms of polymerase, labor and time, and should greatly simplify the development of complex multiplex PCRs to be used in new diagnostic tests.

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Definitions:

1. "Amplification" of DNA as used herein denotes the use of polymerase chain reaction (PCR) to increase the concentration of a particular DNA sequence within a mixture of DNA sequences. An "amplicon" is a target DNA sequence that is amplified by PCR.

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2. "Multiplex PCR" as used herein refers to the simultaneous amplification of multiple DNA targets in a single polymerase chain reaction (PCR) mixture.

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3. "High-throughput" denotes the ability to simultaneously process and screen a large number of DNA samples (e.g. in excess of 100 genomic DNAs) in a rapid and economical manner, as well as to simultaneously screen large numbers of different genetic loci within a single DNA sample.

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4. " T_m " as used herein, refers to the melting temperature (temperature at which 50% of the oligonucleotide is a duplex) of the oligonucleotide calculated using the nearest-neighbor thermodynamic values

of Breslauer et al. (*Proc. Natl. Acad. Sci. USA* 83:3746-3750, 1986) for DNA and Freier et al. (*Proc. Natl. Acad. Sci. USA* 83:9373-9377, 1986) for RNA.

5 5. "ΔG" as used herein, refers to the free
energy for the oligonucleotide as calculated by the nearest
neighbor method of Breslauer et al. (DNA) and Freier et al.
(RNA). The free energy is calculated by the formula:
G = H - TS; where H is the enthalpy, S is the entropy and T
is the temperature. The free energy is a measure of
stability, the greater the negative value, the more stable
the duplex formed by the oligonucleotide.

15 The present invention encompasses methods and
compositions that allow the efficient and essentially
simultaneous amplification of different target DNA
sequences in a single polymerase chain reaction (i.e.,
multiplex PCR). Preferably, equivalent amounts of each
20 amplification product are obtained. The method utilizes
novel chimeric oligonucleotide primers that circumvent the
technical difficulties associated with multiplex PCR that
result in unequal amplification of different target
sequences in the same reaction mix.

25 For example, in a standard PCR reaction employing
more than a single pair of oligonucleotide primers, the
obligatory imposition of a single set of reaction
conditions generally means that one of the primer sets will
function more efficiently in priming, causing the target
30 sequence specified by that set of primers to be
selectively amplified in the early cycles of amplification.
Furthermore, the more efficient primers will also be
depleted from the reaction sooner than the less efficient
ones, resulting in the increased accumulation of non-
35 specific amplification products in later cycles of
amplification. Clearly, these problems are magnified when

it is desired to use multiple primer pairs (>3-4) in a single reaction.

5 The methods and compositions of the present invention circumvent these problems by imposing a uniformly high degree of specificity on the annealing reactions that occur between different primers present in the mixture and their cognate target sequences in the DNA template.

10 During the early cycles of amplification, products are synthesized that contain the chimeric primers on either end. The chimeric primers then serve as high stringency recognition sequences for subsequent rounds of amplification. This results in normalizing the annealing efficiency of different primers and their cognate target

15 sequences, and thus also normalizes the degree of amplification of different targets.

Primer Design

20 Multiplex PCR according to present invention utilizes chimeric oligonucleotide primers that include two domains. The 5' "half" of each primer may comprise any sequence between 17 and 25 bases in length that is unrelated to the target DNA, and has the property of forming hybrids with relatively high melting temperatures (e.g., $T_m > 60^\circ\text{C}$ in the absence of other sequences). In some applications, when the target DNA sequence is embedded in a sequence of low complexity (i.e., <108 bp), primers may be used that form hybrids with lower melting

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30 temperatures.

35 In one embodiment, the 5' sequence comprises 5'-GCGGTCCCAAAAGGGTCAGT-3'. This sequence, which is designated as a "universal primer sequence" (UPS), is derived from the bacteriophage vector M13mp18 (Messing J., *Meth. Enzymol.* 101:20, 1983). Alternative embodiments of invention universal primer sequences for the 5' sequence

comprise the following oligonucleotides:

5 #1: 5'-CGC CAG GGT TTT CCC AGT CA-3'
 #2: 5'-CGC CAG GGG GGG CCC AGT CA-3'
 #3: 5'-CGG CAG CGG GGC CCA GTC CA-3'
 #4: 5'-CGC GGC CGG GGC CCA TCC CA-3'
 #5: 5'-CGC GGC CGG GGC CAT CTC AA-3'
 #6: 5'-GAG GCC GGT GGC CAT GTC AA-3'
 #7: 5'-TAG GCG CGT GGC CAT GTC AA-3'
 #8: 5'-TAG GCC CGT GGC CAT GTT AA-3'
 #9: 5'-TAG GCC CGT GGC AAT ATT AA-3'
 #10: 5'-CCG GTC CGT GGC AAT ATT AA-3'
 #11: 5'-CCG GCT CGT GGC GAT GTT AA-3'
 #12: 5'-CCG GCG TGT GCC GAT ATT AA-3'
15 #13: 5'-CCA TGC GTG TGC CGA TAT TA-3'
 #14: 5'-CAA TGC GGG CGC CGA TAT TA-3'
 #15: 5'-CGA TGC GGG AGC CAA TAT AA-3'
 #16: 5'-AGA TGC GGT AGC CAA TAT AA-3'
 #17: 5'-GGC GTG CTG AGC CAA TAT GG-3'
20 #18: 5'-GGC GCG CTG AGC CAA TAT GG-3'
 #19: 5'-GGC GCG CCG AGC CAA TAT GG-3'
 #20: 5'-GGC GCG CCG AGC TAA TAT AT-3'
 #21: 5'-AGC TCG GCG AGC TAA TAT AT-3'
 #22: 5'-AGC GCG GCC AGC TAA GAG AT-3'
25 #23: 5'-CGC GCG GCC GGC TGG AGA GA-3'
 #24: 5'-CGC GAG GCC GGC TGT AGA GG-3'
 #25: 5'-CGC GAG GCC AGC GGC CGA GG-3'
 #26: 5'-CGC GAG GCC AGC GGT CGA GG-3'
 #27: 5'-CGC GAG GCC AGC GGT CGA GG-3'
30 #28: 5'-CGC GGG GCC CGC GGC CGC GG-3'
 #29: 5'-CGC CCG CCG CGC CCC GCG CC-3'
 #30: 5'-GGC GCT CCA TTA GCG TGA GT-3'

35 The 3' "half" of each primer comprises a target-specific sequence, i.e., a sequence that is either present or potentially present in the target DNA or its complement.

These 3' sequences may comprise without limitation any such sequence of 17-25 bases, and preferably 20 bases, irrespective of the melting temperatures of hybrids formed between the isolated sequence and its complement.

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In one embodiment, the 3' half of the primer is intended to hybridize with a genomic sequence flanking the target sequence of interest; in this case, the primer is used to amplify the target sequence for subsequent diagnostic tests such as, e.g., hybridization with allele-specific oligonucleotides, restriction endonuclease cleavage, or single-strand conformational polymorphism (SSCP) analysis. For this purpose, the 3' half of the primer must correspond to a sequence known to be present in all DNA samples to be tested (or its complement). Non-limiting examples of 3' primer halves useful in practicing the present invention are shown in Figure 1.

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In another embodiment, the amplification reaction itself serves as the critical diagnostic step. In this case, the 3' sequence of the primer corresponds to a defined wild-type version of a particular amplicon or its complement (or to a variant version or its complement) whose presence or absence is being tested. When such allele-specific sequences are incorporated into chimeric PCR primers according to the present invention, and the chimeric primers are used in amplification reactions, the absence of a given amplification product is considered definitive for the absence of the allele in the DNA sample being tested.

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For use in a given multiplex PCR reaction, target-specific primer sequences are typically analyzed as a group to evaluate the potential for fortuitous dimer formation between different primers. This evaluation may be achieved using commercially available computer programs for sequence analysis, such as Gene Runner, Hastings

Software Inc. Other variables, such as the preferred concentrations of Mg⁺⁺, dNTPs, polymerase, and primers, are optimized using methods well-known in the art (Edwards et al., *PCR Meth. App.* 3:565, 1994).

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DNA templates

Any DNA sample may be used in practicing the present invention, including without limitation eukaryotic, prokaryotic and viral DNA. In a preferred embodiment, the target DNA represents a sample of genomic DNA isolated from a patient. This DNA may be obtained from any cell source or body fluid. Non-limiting examples of cell sources available in clinical practice include blood cells, buccal cells, cervicovaginal cells, epithelial cells from urine, fetal cells, or any cells present in tissue obtained by biopsy. Body fluids include blood, urine, cerebrospinal fluid, semen and tissue exudates at the site of infection or inflammation. DNA is extracted from the cell source or body fluid using any of the numerous methods that are standard in the art. It will be understood that the particular method used to extract DNA will depend on the nature of the source. The preferred amount of DNA to be extracted for use in the present invention is at least 5 pg (corresponding to about 1 cell equivalent of a genome size of 4 x 10⁹ base pairs).

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Multiplex PCR reaction conditions

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In practicing the present invention, a DNA sample is contacted with pairs of chimeric oligonucleotide primers under conditions suitable for polymerase chain reaction. Standard PCR reaction conditions may be used, e.g., 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 200 μM deoxynucleotide triphosphates (dNTPs), and 25-100 U/ml Taq polymerase (Perkin-Elmer, Norwalk, CT).

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The concentration of each chimeric primer in the reaction mixture can range from about 0.05 to about 4 μ M. The optimal concentration for primer is evaluated by performing single PCR reactions using each primer pair individually. Similarly, each primer pair is evaluated independently to confirm that all primer pairs to be included in a single multiplex PCR reaction require the same amplification conditions (i.e., temperature, duration of annealing and extension steps). It was found (see example below) that all chimeric primers containing the M13 derived UPS as the 5' half of their sequence could be used at a broad range of annealing temperatures (i.e., 50-60°C).

15 Multiplex PCR reactions are carried out using
manual or automatic thermal cycling. Any commercially
available thermal cycler may be used, such as, e.g.,
Perkin-Elmer 9600 cycler.

20 Finally, the reaction products are analyzed using
any of several methods that are well-known in the art.
Preferably, agarose gel electrophoresis is used to rapidly
resolve and identify each of the amplified sequences. In a
multiplex reaction, different amplified sequences are
25 preferably of distinct sizes and thus can be resolved in a
single gel. In one embodiment, the reaction mixture is
treated with one or more restriction endonucleases prior to
electrophoresis. Alternative methods of product analysis
include without limitation dot-blot hybridization with
30 allele-specific oligonucleotides and SSCP.

The following examples are intended to further illustrate the present invention without limiting the invention thereof.

Example 1: Primer design:

5 Sequence-specific primers were chosen without regard to hairpin formation and having a calculated ΔG for primer duplexing below -10 kcal/mole. The T_m of these primers range from 52° to 68° C as determined by the A+T/G+C method. To evaluate potential primer dimer formation within a primer set, primers sets were analyzed
10 using Amplify 1.2 software (University of Wisconsin, Department of Genetics, Madison, WI). The universal primer sequence (UPS) 5' GCGGTCCCAAAAGGGTCAGT 3' is from bacteriophage M13mp18.

15 The UPS-tagged primers contain the 20 nucleotide UPS sequence attached to the 5' end of the individual sequence-specific primers listed in Figure 1.

20 Oligonucleotide primers were synthesized by Operon Technologies (Alameda, CA). Oligonucleotides were HPLC purified and quantitated by spectrophotometry.

Example 2: DNA preparation:

25 Whole blood samples were collected in high glucose ACD Vacutainers™ (Beckton Dickenson & Co., Franklin Lanes, NJ). Following centrifugation, the buffy coat was collected and lysed with two washes of a 10:1 (v/v) solution of 14 mM NH₄Cl and 1 mM NaHCO₃. The
30 lymphocytes were harvested by centrifugation, resuspended in lysis buffer (10 mM Tris-HCl, pH 8.0, 0.4 M NaCl, 2 mM EDTA, 0.5% SDS, 500 µg/ml proteinase K) and incubated overnight at 37°C. Samples were then extracted with 1/4th volume of saturated NaCl, and the DNA was collected by
35 ethanol precipitation. The final DNA pellet was washed

with 70% ethanol, air dried and dissolved in TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA).

5 Buccal cell samples were obtained by brushing the lining of the buccal cavity for 30 seconds with a sterile cytology brush (Scientific Products #S7766-1a). DNA was prepared by immersing the brushes in 600 μ l of 50 mM NaOH in 1.2 ml 96-well polypropylene tubes (USA/Scientific Plastics, Ocala, Florida) and vortexed. The tubes, still containing the brushes, were heated to 95° C for 5 min. and the brushes were carefully removed. The lysates were neutralized with 60 μ l of 1 M Tris-HCl (pH 8.0) and vortexed. Samples were stored at 4° C.

15 Example 3: Amplification reactions:

20 Single amplifications were performed using 4 μ l (1-2 μ g) and 10 μ l (5-50ng) of genomic DNA prepared (described above) from either blood or buccal cells, respectively. 50 μ l reaction mixtures were carried out in 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂), 200 μ M dNTPs, and 2.5 units Taq polymerase (Perkin-Elmer, Norwalk, CT).

25 Multiplex PCR reactions were carried in a volume of 100 μ l under the same conditions, except that 10 units of Taq polymerase per reaction was used.

30 To demonstrate the use of tagged primers for multiplex PCR, the 32 primer pairs presented in Figure 1 were employed either alone, or as chimeric primers containing a 20 nucleotide tag (GCGGTCCCAAAAGGGTCAGT) corresponding to the M13mp18 universal primer sequence (UPS) at the 5' end. The T_m for all of the chimeric 35 primers was calculated to be greater than 72° C.

Primer concentrations ranged from 0.25 to 1.0 μ M. Amplifications were carried out using a Perkin-Elmer 9600 thermocycler (Perkin-Elmer, Norwalk, CT) for 28 cycles with ramping (melting at 94°C for 10s, annealing at 50°C, 55°C, 60°C, or 65°C for 10 s, and extension at 72°C for 10s).

After completion of the reaction, 8 μ l of the reaction products were loaded directly onto a 2% ethidium bromide-stained agarose gel and subjected to electrophoresis at 250 volts for 90 minutes. The amplification products were visualized with a UV transilluminator and photographed with an Alpha Innotech IS-500 Digital Imaging System version 1.97 (Sun BIO Science, Inc., Branford, CT).

The following experiments were performed to demonstrate the effects on amplification of incorporating invention primer sequences into PCR primers.

Example 4: Effect of Chimeric Primers on Efficiency and Specificity of Amplification

Three sequence-specific primer pairs used to amplify Exon 21 of the cystic fibrosis transmembrane regulator (CFTR) gene (Kerem et al., *Science* 245:1073, 1989) are shown in Figure 2. A chimeric version of one of the three primers was synthesized containing the M13 UPS sequence 5'-GCGGTCCCAAAAGGGTCAGT-3' immediately 5' to the illustrated sequences. The oligonucleotides were synthesized using conventional chemistry and were purified by high-performance liquid chromatography prior to use.

Primers were serially diluted two-fold for each primer pair (SS#1, SS#2, SS#3, and SS#3+UPS. See Figure 1). Primer pairs SS#1, SS#2 and SS#3 are sequence specific primers only; SS#3 + UPS is chimeric with the 5' UPS sequence.

The results show that the addition of the UPS tag confers enhanced specificity over a four fold range of primer concentrations while retaining amplification efficiency at annealing temperatures from 50° to 65° C. Based on this and similar analyses of additional chimeric primer pairs, an annealing temperature of 60° C was determined to be optimal for PCR amplification using UPS-tagged primers.

The efficiencies with which the three CFTR primer pairs (designated SS#1, SS#2, and SS#3) primed amplification varied with primer concentration and temperature of annealing (Figure 2). The primer concentrations were as follows: Lanes 1, 4, 7, and 10, (1.0 μ M); lanes 2, 5, 8, and 11, (0.5 μ M); and lanes 3, 6, 9, and 12, (0.25 μ M). The temperatures of annealing were 50°C, 55°C, 60°C and 65°C, as indicated.

The SS#1 and SS#3 primers, for example, were noticeably inefficient at annealing temperatures above 60°C. The primer pair designated SS#3-UPS, which corresponds to the SS#3 primers having the M13 UPS sequence on their 5' termini, was highly efficient in priming at all temperatures tested; furthermore, few spurious amplification products were detected in reactions containing SS#3-UPS primers. By contrast, SS#2 primers gave spurious amplification products at all three temperatures below 65°C.

Example 5: Multiplex Amplification with Chimeric Primers

To assess the use of chimeric primers for multiplex PCR, a system was designed using 15 UPS-tagged primer pairs specific for the cystic fibrosis transmembrane conductance regulator locus (CFTR). An example of the gel

electrophoretic pattern of the multiplex PCR products is shown in Figure 3.

As demonstrated for exon 21 (Figure 2), each
5 chimeric primer pair concentration used within the CFTR
15-plex was determined by performing independent amplicon
amplifications over a range of concentrations. Lanes 1-3;
10 multiplex amplification of 3 different genomic DNA samples
isolated from blood. All samples were amplified with CFTR
chimeric 15-plex primers (See Figure 1) at the following
primer pair concentrations: Int 19; 0.5 μ M. exon 19; 0.5 μ M.
exon 21; 1.0 μ M. exon 9; 0.75 μ M. exon 13; 1.0 μ M. exon 4;
15 1.0 μ M. exon 17b; 0.5 μ M. exon 7; 0.5 μ M. exon 11; 1.0 μ M. exon
exon 10; 1.0 μ M. exon 20; 0.25 μ M. exon 5; 0.5 μ M. exon 14b; 0.5 μ M.
exon 12; 0.5 μ M. exon 3; 0.25 μ M. Lane M; Φ X174 Hae III
digested marker DNA.

Using the same amplification conditions that were
originally defined for the individual chimeric primer
20 pairs, all 15 of the predicted CFTR PCR products
co-amplified with relative ease. It should be emphasized
that the CFTR multiplex reactions presented in Figure 3 and
all subsequent reactions employed the same reaction
components, cycling times and temperatures without
25 modification from the single amplicon assay
conditions. The only optimization required was an
adjustment of the primer concentrations for intron 19 and
exon 19 to generate comparable band intensities of the
co-amplified products.

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Example 6: Comparison of Multiplex PCR Reactions Using
CFTR Primer Pairs Lacking and Containing M13 UPS

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To demonstrate the enhanced specificity and
efficiency conferred by the UPS sequence in multiplex PCR,
parallel reactions were carried out using UPS-tagged CFTR

primers and the corresponding non-tagged primers. Identical reaction conditions, cycling times and primer concentrations were used for both primer sets. Previously, optimal primer concentrations were determined for the individual primer pairs. Coincidentally, the optimal concentrations determined in this manner were identical for both the UPS and non-UPS tagged primers.

Figure 4 shows the results of agarose gel comparison of chimeric primers with sequence-specific primers for CFTR 15-plex PCR amplification. Lanes 1-8; genomic DNA samples isolated from blood. Lanes 9-16; genomic DNA samples isolated from buccal cells. Each consecutive sample in the group of four amplifications represents a two-fold serial dilution of genomic DNA. Lanes 1-4 and 9-12; CFTR 15-plex amplification with chimeric primers. Lanes 5-8 and 13-16; CFTR 15-plex amplification with sequence-specific primers. Lane M; Φ X174 Hae III digested marker DNA.

As shown, the multiplex amplification using the standard sequence-specific primer pairs failed to generate a clear multiplex PCR profile of the CFTR locus. Specifically, several of the expected bands were clearly under-represented, presumably due to differential PCR amplification of their respective products (Figure 4, lanes 5-8 and lanes 13-16).

In contrast, a clear multiplex profile was obtained when the CFTR locus was amplified with the corresponding UPS-tagged primer pairs. The expected bands were clearly prominent and virtually free of contaminating products (Figure 4, lanes 1-4 and 9-12). Moreover, equivalent banding patterns were observed over an 8-fold range of template concentrations when UPS-tagged primer pairs were employed.

Conversely, the amplification profile generated using sequence-specific primers was sensitive to variations in the template concentration as evident by the changes in the intensity of individual bands (Figure 4, lanes 5-8 and 13-16).

Example 7: Use of Multiplex PCR to Simultaneously Amplify Different Disease-related Sequences Under Identical Conditions

To further demonstrate the general utility of the UPS-tagged primers, 29 different human genomic target sequences (Figure 1) were amplified in a single thermal cycler under identical reaction conditions and cycling parameters. Results from these amplification reactions using sequence-specific primer pairs and the cognate UPS-tagged primer pairs are presented in Figure 5 and Figure 6.

The banding patterns for the following primer sets are displayed in Figure 5: lanes 1-3 amplification of CFTR 15-plex with chimeric primers; lanes 4-6 amplification of CFTR 15-plex with sequence-specific primers; lanes 7-9 multiplex amplification of α -galactosidase Gaucher's disease (GCR) (Kornreich et al., *Nuc. Acids Res.* 17:3301, 1989) 3-plex and single Sickle Cell Anemia (SCA) (Navon et al., *Science* 243:1471, 1989) target with chimeric primers; lanes 10-12 multiplex amplification of GCR 3-plex and single SCA target with sequence specific primers; lanes 13-15 multiplex amplification of single GCR target and Tay-Sachs (TS) (Tanaka et al., *Am. J. Hum. Genet.* 46:329, 1990) 2-plex with chimeric primers; lanes 16-18 multiplex amplification of single GCR target and TS 2-plex with sequence-specific primers; lanes 19-21 amplification of single β -thalassemia target with chimeric primers; and

lanes 22-24 amplification of single β -thalassemia target with sequence-specific primers. Lane M; Φ X174 Hae III digested marker DNA. All primer pairs were tested on 3 different genomic DNA samples isolated from: lanes 1-8 genomic DNA samples isolated from blood cells, while in lanes 9-12 the genomic DNA template was derived from buccal cells.

Figure 6 shows the results of independent amplification of six target sequences within the human Wilms Tumor gene (WT1) (Varanasi et al., *Proc. Natl. Acad. Sci. USA* 91:3554, 1994). Lanes 1, 2, 5, 6, 9, 10, 13, 14, 17, 18, 21 and 22; amplifications performed with chimeric primers. Lanes 3, 4, 7, 8, 11, 12, 15, 16, 19, 20, 23 and 24; amplifications performed with sequence-specific primers. Amplifications represent each of six amplicons within the WT-1 gene (B, F, II, J, N, and O; see Figure 1). All primer pairs tested on two independent genomic DNA samples isolated from blood. Lane M; Φ X174 Hae III digested marker DNA. With the exception of one primer pair (Figure 6, lanes 5-8) which did not generate a detectable product when the chimeric primer was employed, the presence of the UPS sequence enhanced the yield of the respective PCR products (Figure 6, lanes 1-4, 9-12, 13-16, 17-20, 21-24).

Results from the multiplex PCR reactions presented herein demonstrate that for both single gene multiplexes (Figure 5, lanes 1-3) and multiplex reactions involving more than one gene (Figure 5, lanes 7-9, 13-15), the UPS-tagged primers generated only the desired bands, and the co-amplified products were more uniform with respect to the band intensities than the corresponding products generated from the non-tagged sequence-specific primers (Figure 6, lanes 4-6, 10-12, 16-18).

What is Claimed is:

1. A single-stranded oligonucleotide DNA primer for amplification of a target DNA sequence capable of use in a multiplex polymerase chain reaction (PCR), said primer having the structure 5'-XY-3', wherein

5 a) X comprises a sequence that does not hybridize to said target sequence;

b) the melting temperature of a hybrid between X and its complement in the absence of other sequences is greater than about 60°C; and

10 c) Y comprises a sequence contained within or flanking said target sequence or its complement.

2. The primer of claim 1, wherein X comprises the sequence 5'-GCGGTCCCCAAAAGGGTCAGT-3'.

3. The primer of claim 1, wherein X comprises the sequence 5'-CGCCAGGGTTTCCCAGTCA-3'.

4. The primer of claim 1, wherein X comprises the sequence 5'-CGCCAGGGGGGGCCCAGTCA-3'.

5. The primer of claim 1, wherein X comprises the sequence 5'-CGGCAGCGGGGCCAGTCCA-3'.

6. The primer of claim 1, wherein X comprises the sequence 5'-CGGGGCCGGGGCCCATCCCA-3'.

7. The primer of claim 1, wherein X comprises the sequence 5'-CGGGCCGGGGCCATCTCAA-3'.

8. The primer of claim 1, wherein X comprises the sequence 5'-GAGGCCGGTGGCCATGTCAA-3'.

9. The primer of claim 1, wherein X comprises the sequence 5'-TAGGCGCGTGGCCATGTCAA-3'.

10. The primer of claim 1, wherein X comprises the sequence 5'-TAGGCCCGTGGCCATGTTAA-3'.

11. The primer of claim 1, wherein X comprises the sequence 5'-TAGGCCCGTGGCAATATTAA-3'.

12. The primer of claim 1, wherein X comprises the sequence 5'-CCGGTCCGTGGCAATATTAA-3'.

13. The primer of claim 1, wherein X comprises the sequence 5'-CCGGCTCGTGGCGATGTTAA-3'.

14. The primer of claim 1, wherein X comprises the sequence 5'-CCGGCGTGTGCCGATATTAA-3'.

15. The primer of claim 1, wherein X comprises the sequence 5'-CCATGCGTGTGCCGATATTA-3'.

16. The primer of claim 1, wherein X comprises the sequence 5'-CAATGCGGGCGCCGATATTA-3'.

17. The primer of claim 1, wherein X comprises the sequence 5'-CGATGCGGGAGCCAATATAA-3'.

18. The primer of claim 1, wherein X comprises the sequence 5'-AGATGCGGTAGCCAATATAA-3'.

19. The primer of claim 1, wherein X comprises the sequence 5'-GGCGTGCTGAGCCAATATGG-3'.

20. The primer of claim 1, wherein X comprises the sequence 5'-GGCGCGCTGAGCCAATATGG-3'.

21. The primer of claim 1, wherein X comprises the sequence: 5'-GGCGCGCCGAGCCAATATGG-3'.

22. The primer of claim 1, wherein X comprises the sequence 5'-GGCGCGCCGAGCTAATATAT-3'.

23. The primer of claim 1, wherein X comprises the sequence 5'-AGCTCGGCGAGCTAATATAT-3'.

24. The primer of claim 1, wherein X comprises the sequence 5'-AGCGCGGCCAGCTAAGAGAT-3'.

25. The primer of claim 1, wherein X comprises the sequence 5'-CGCGCGGCCGGCTGGAGAGA-3'.

26. The primer of claim 1, wherein X comprises the sequence 5'-CGCGAGGCCAGCGGCTGAGAGG-3'.

27. The primer of claim 1, wherein X comprises the sequence 5'-CGCGAGGCCAGCGGCCGAGG-3'.

28. The primer of claim 1, wherein X comprises the sequence 5'-CGCGAGGCCAGCGGTAGAGG-3'.

29. The primer of claim 1, wherein X comprises the sequence 5'-CGCGAGGCCAGCGGTAGAGG-3'.

30. The primer of claim 1, wherein X comprises the sequence 5'-CGCGGGGCCCGCGCCCCGCGGG-3'.

31. The primer of claim 1, wherein X comprises the sequence 5'-CGCCCGCCGCGCCCCGCGCC-3'.

32. The primer of claim 1, wherein X comprises the sequence 5'-GGCGCTCCATTAGCGTGAGT-3'.

33. The primer of claim 1, wherein X and Y each comprise from 17 to 20 bases.

34. The primer of claim 1, wherein the melting temperature of a hybrid formed between said primer and its complement in a solution of 0.5M NaCl is at least 72°C.

35. An oligonucleotide DNA primer for amplification of a target DNA sequence, wherein said primer consists of the sequence 5'-GCGGTCCCAAAAGGGTCAGT[Y]-3', wherein Y comprises a sequence contained within or flanking said target sequence or its complement.

36. A method for simultaneous amplification of multiple DNA target sequences present in a DNA sample, which comprises:

5 a) contacting said DNA sample in a single reaction mixture with a multiplicity of paired oligonucleotide primers having the structure 5'-XY-3', wherein

(i) X comprises the sequence 5'-GCGGTCCCAAAAGGGTCAGT-3', and

10 (ii) Y comprises a sequence contained within or flanking said target sequence or its complement; and

b) performing multiple cycles of melting, reannealing, and DNA synthesis.

37. A method for detecting multiple defined target DNA sequences in a DNA sample, which comprises the steps of:

5 a) contacting said DNA sample in a single reaction mixture with a multiplicity of oligonucleotide pairs, each of said pairs consisting of a first and second oligonucleotide primer, wherein

(i) said first primer of each pair has the structure 5'-XY-3', wherein X comprises the

10 sequence 5'-GCGGTCCCAAAAGGGTCAGT-3' and Y comprises a sequence contained within the target sequence or its complement, and
(ii) said second primer of each pair has the structure 5'-XY-3', wherein X comprises the sequence 5'-GCGGTCCCAAAAGGGTCAGT-3', and Y comprises a sequence flanking the target sequence or its complement;

15 b) performing multiple cycles of melting, re-annealing, and DNA synthesis to form amplification products of DNA samples primed with said oligonucleotides; and
20 c) detecting the amplification products.

38. The method of claim 37 wherein detection of an amplification product indicates the presence of the target sequence in the DNA sample.

39. The method of claim 37 wherein said detecting step comprises gel electrophoresis.

40. A method for high-throughput genetic screening to simultaneously detect the presence of multiple defined target DNA sequences in DNA samples obtained from a multiplicity of individuals, said method comprising the steps of:

5 a) providing a sample of DNA from each of said individuals;

b) simultaneously contacting each of said DNA samples obtained in a) with a multiplicity of 10 oligonucleotide pairs, each of said pairs consisting of a first and second oligonucleotide primer, wherein

15 (i) said first primer of each pair has the structure 5'-XY-3', wherein X comprises the sequence 5'-GCGGTCCCAAAAGGGTCAGT-3' and Y comprises a sequence contained within the target sequence or its complement, and

20

(ii) said second primer of each pair has the structure 5'-XY-3', wherein X comprises the sequence 5'-GCGGTCCCAAAAGGGTCAGT-3', and Y comprises a sequence flanking the target sequence or its complement;

25

c) performing multiple cycles of melting, re-annealing, and DNA synthesis to form amplification products; and

d) detecting the amplification products.

41. The method of claim 40 wherein detection of an amplification product indicates the presence of the target sequence in the DNA sample.

42. The method of claim 40 wherein said detecting step comprise gel electrophoresis.

Cystic Fibrosis Transmembrane Regulator (CFTR) Primer Sequences	Exon Int 19	15-plex Size (bp) -440	Gauchers (GCR) and Sickle Cell Anemia (SCA) 4-plex		
			Exon 6	Exon 6	Size (bp) 401
AGG CTT CTC AGT GAT CTG TTG GAA TCA TTC AGT GGG TAT AAG CAG	19	410	GAA TGT CCC AAG CCTT TIG A AAG CTG AAG CAA GAG AAT CG	2	358
GGC CGA CAA ATA ACC AAG TGA AGT CTA ACA AAG GAA GCA GTG	21	381	TGC AAC TAC TGA GGC ACT T TAC AAT GAT GGG ACT GTC G	9	319
CTT CTA ATG GTG ATG ACA GGC T CCA CTG AAA ATA ATA TGA GGA AAT	9	335	SCA Primer Sequences CAT TTG CTT CTG ACA CAA CTG CCA ACT TCA TCC ACG TTC ACC	124	
AGG TAG CAG CTA TTT TTA TGG TAA GGG AGT CTT TTG CAC AA	13	295	GCR and Tay-Sachs (TS) 3-plex GCR CCT TGC CCT GAA CCC CGA A CTG ACT CTG TCC CTT TAA TGC CCA	1 / 6	
TGT AGG AAG TCA CCA AAG CGA TAC AGA ATA TAT GTG CC	4	267	TS Primer Sequences GGA TGC CAA TTT TCA CTC ATC TTG AGT TAA TGA GTT CAT AGT ACC TGT T	9, 10, 11	871
GGG GTC CAA TTT TCA CTC ATC TTG AGA TAC TTC AAT AGC TCA GGC TT	17b	245	TS Primer Sequences GTC TGG CGA GAG GAT ATT CCA TGG CTA GAT GGG ATT GGG TCT	11, 12***	530
GGT ACA TTA CCT GTC TTT TGT TT	7	220	GGG TCC TAC AAC CCT GTC ACC CAC AAG CTT CAC TCT GAG CAT AAC AAG	7***	190
CAG ATT GAG CAT ACT AAA AGT G TAC ATG AAT GAC ATT TAC AGC A	11	200	B-thalassemia Primer Sequences GCT GTC ATC ACT TAG ACC TC GCA AGA AAG CGA GCT TAG TG	1, 2, 3	1612
GAG CCT TCA GAG GGT AAA AT	10	175			

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FIG. IA

50°

55°

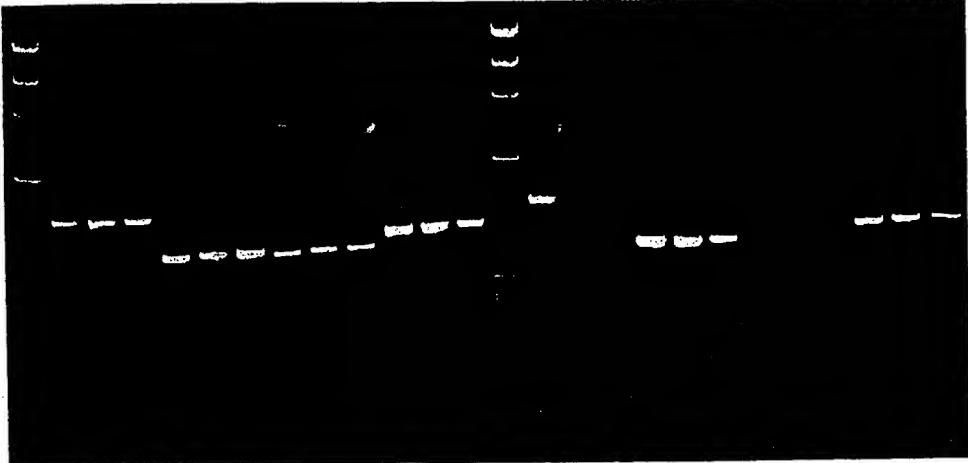
SS#1	SS#2	SS#3	SS#3+UPS	SS#1	SS#2	SS#3	SS#3+UPS
M 1 2 3 4 5 6 7 8 9 10 11 12	M 1 2 3 4 5 6 7 8 9 10 11 12						

**FIG. 2A****FIG. 2B**

60°

65°

SS#1	SS#2	SS#3	SS#3+UPS	SS#1	SS#2	SS#3	SS#3+UPS
M 1 2 3 4 5 6 7 8 9 10 11 12	M 1 2 3 4 5 6 7 8 9 10 11 12						

**FIG. 2C****FIG. 2D****SUBSTITUTE SHEET (RULE 26)**

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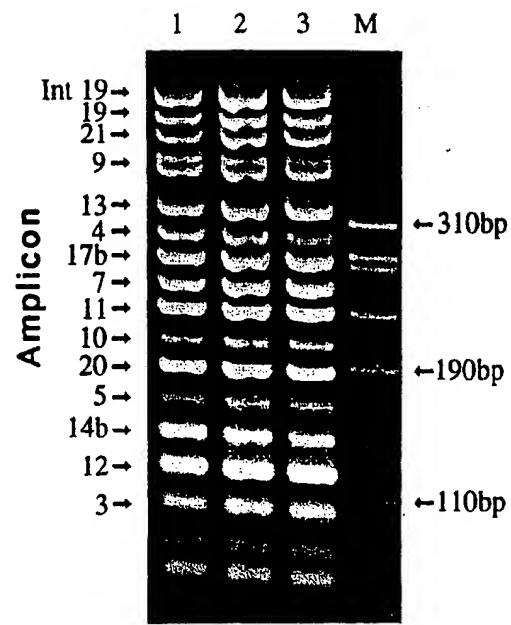


FIG. 3

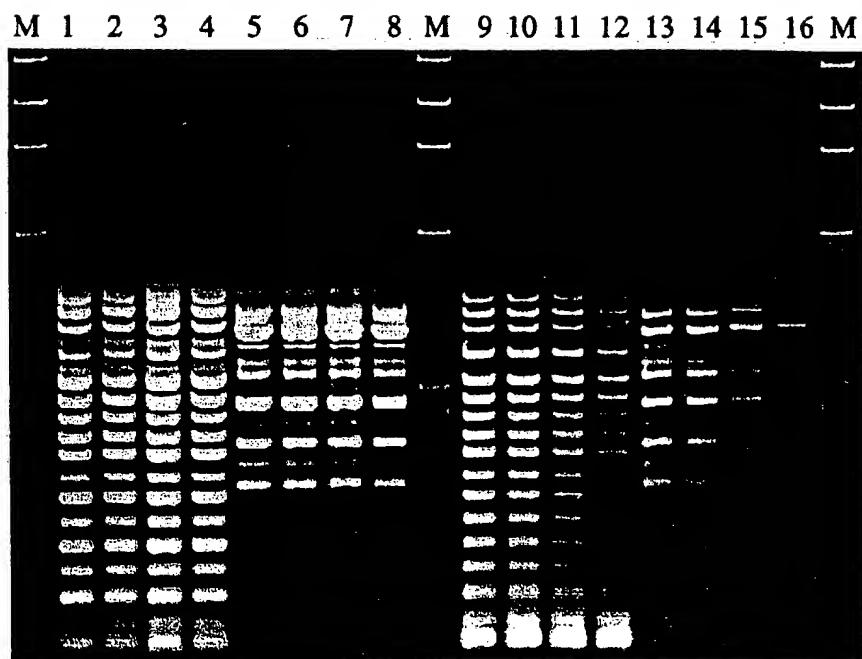
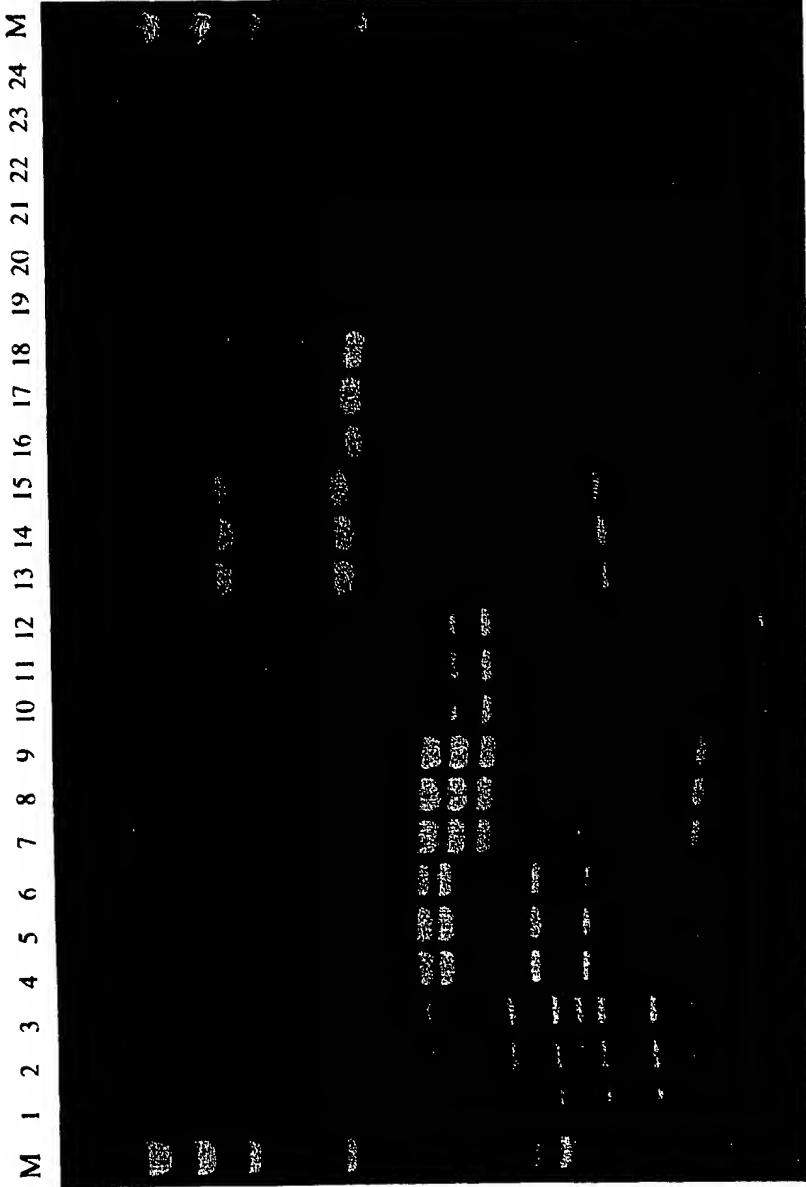


FIG. 4

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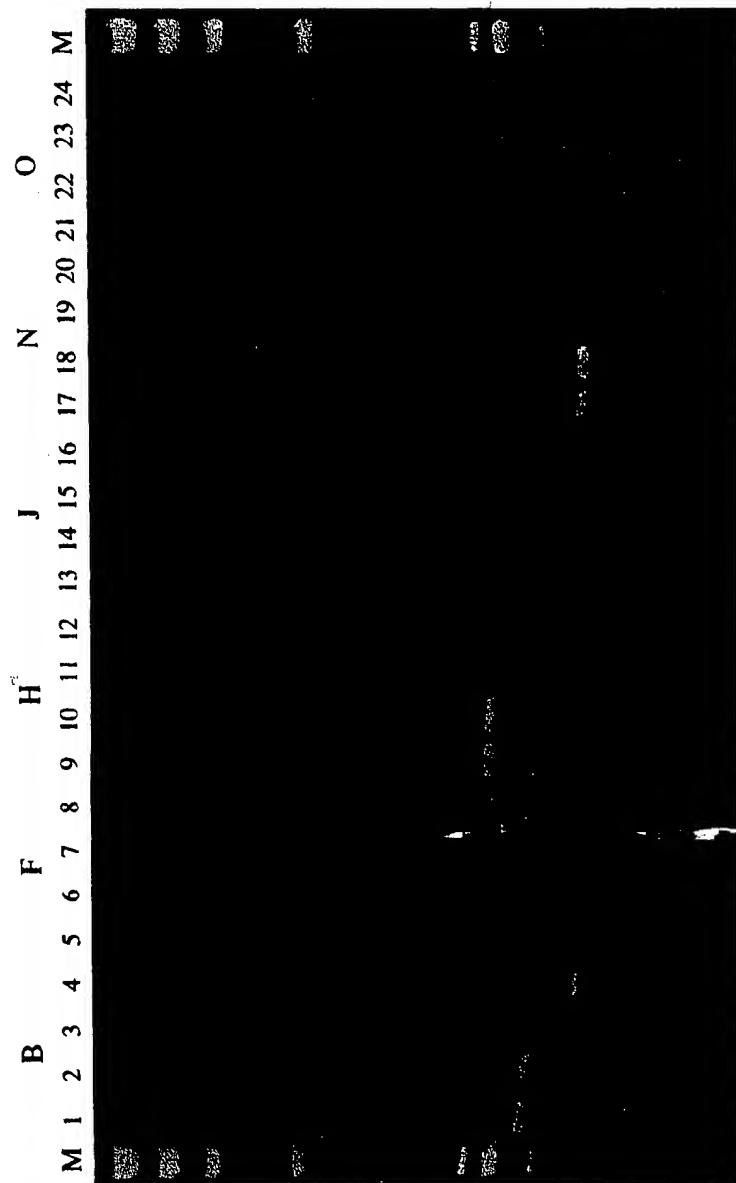


FIG. 6

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US 96/09637

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C07H21/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	US,A,5 104 792 (SILVER JONATHAN ET AL) 14 April 1992 see the whole document ---	1-42
A	PCR METHODS AND APPLICATIONS, vol. 3, no. 1, August 1993, CSH LABORATORY PRESS, CSH, NEW YORK, US, pages 77-80, XP000609125 F. WEIGHARDT ET AL.: "A simple procedure for enhancing PCR specificity" cited in the application see the whole document ---	1-42

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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Date of the actual completion of the international search

Date of mailing of the international search report

13 November 1996

18.11.96

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Hornig, H

INTERNATIONAL SEARCH REPORT

International application No
PCT/US 96/09637

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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WO-A-9318177	16-09-93	NONE		
WO-A-9318178	16-09-93	NONE		